14-3-3 (Bmh) Proteins Inhibit Transcription Activation by Adr1 through Direct Binding to Its Regulatory Domain[▽]

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14-3-3 proteins, known as Bmh in yeast, are ubiquitous, highly conserved proteins that function as adaptors in signal transduction pathways by binding to phosphorylated proteins to activate, inactivate, or sequester their substrates. Bmh proteins have an important role in glucose repression by binding to Reg1, the regulatory subunit of Glc7, a protein phosphatase that inactivates the AMP-activated protein kinase Snf1. We describe here another role for Bmh in glucose repression. We show that Bmh binds to the Snf1-dependent transcription factor Adr1 and inhibits its transcriptional activity. Bmh binds within the regulatory domain of Adr1 between amino acids 215 and 260, the location of mutant *ADR1ᶜ* alleles that deregulate Adr1 activity. This provides the first explanation for the phenotype resulting from these mutations. Bmh inhibits Gal4-Adr1 fusion protein activity by binding to the Ser230 region and blocking the function of a nearby cryptic activating region. *ADR1ᶜ* alleles, or the inactivation of Bmh, relieve the inhibition and Snf1 dependence of this activating region, indicating that the phosphorylation of Ser230 and Bmh are important for the inactivation of Gal4-Adr1. The Bmh binding domain is conserved in orthologs of Adr1, suggesting that it acquired an important biological function before the whole-genome duplication of the ancestor of *S. cerevisiae*.

14-3-3 proteins are ubiquitous, highly conserved proteins that have important roles in signal transduction pathways in eukaryotes (1, 28, 29). Their targets are generally Ser- or Thr-phosphorylated motifs of proteins that play a role in nucleo-cytoplasmic signaling and include protein kinases, protein phosphatases, and transcription factors (2, 8, 9, 13, 28, 29, 39, 51, 54, 58, 68, 72, 74). 14-3-3 binding can inhibit or activate enzyme activity, regulate protein localization, and serve as a molecular scaffold or adaptor for other proteins.

In the yeast *Saccharomyces cerevisiae*, there are two genes encoding redundant 14-3-3 proteins, Bmh1 and Bmh2 (reviewed in reference 69). As in higher eukaryotes, Bmh proteins are involved in numerous signaling and cell differentiation pathways, including pseudohyphal differentiation, DNA damage checkpoint, nitrogen catabolism, TOR signaling, stress response, protein degradation, retrograde signaling, exocytosis and vesicle transport, catabolite inactivation, and cell cycle regulation.

Global proteomic analyses of proteins copurifying with an epitope-tagged version of Bmh1 or showing a two-hybrid interaction with Bmh identified more than 60 potential substrates, including several transcription factors (34, 37, 67). One of the transcription factors that copurified with Bmh1 is Adr1, the subject of this report. Bmh proteins regulate the intracellular location of several substrates, but how Bmh might affect the activity of most of its targets is unknown.

Bmh proteins have an important role in glucose repression in *S. cerevisiae* (25, 40). 14-3-3 proteins in animal cells have an analogous role in response to nutrient deprivation by regulat-ing TOR signaling (35). When yeast ferments glucose, the expression of genes involved in the utilization of alternative carbon sources is repressed. After glucose is exhausted, derepression of the transcription of a large number of genes required for respiratory growth takes place (24). Derepression requires phosphorylation and activation of the AMP-activated protein kinase Snf1 by one of three upstream kinases (reviewed in reference 36). Glc7, a type I protein phosphatase, acts in concert with its regulatory subunit Reg1 to keep Snf1 in an inactive, nonphosphorylated state in the presence of high levels of glucose (55). When either Reg1 or Glc7 is inactivated, many glucose-repressed genes are expressed in a constitutive manner due to the phosphorylation and activation of Snf1. Similarly, loss of Bmh activity causes a loss of glucose repression (25, 40).

The two Bmh isoforms copurify with Reg1 (25, 47). Their copurification and the loss of glucose repression in mutants lacking either Reg1 or Bmh activity suggest that Bmh may play an important role in Snf1 inactivation through interactions with Reg1 (25). However, we observed a strong synergism of glucose-resistant *ADH2* expression when both *BMH* genes and *REG1* were deleted, suggesting that Bmh also has a Reg1-independent role in glucose repression (25).

ADH2 is a canonical glucose-repressed gene that is dependent on Snf1 for expression when glucose is exhausted (11, 18, 75). Snf1 activates *ADH2* through two transcription factors, Adr1 and Cat8, which bind cooperatively to adjacent upstream activation sequence elements in the promoter (19, 26, 63, 71, 77). Promoter binding of Adr1 and Cat8 and the consequent Adr1-dependent chromatin remodeling, coactivator recruitment, and preinitiation complex (PIC) formation are glucose repressed and dependent on Snf1 after glucose depletion (3, 4, 70, 76). How Snf1 regulates these activities of Adr1 is unknown.

Adr1 is a transcriptional regulator of other glucose-re-

pressed genes in yeast (75). These target genes include those required for ethanol, glycerol, lactate, and amino acid utilization, peroxisome biogenesis, and the β-oxidation of fatty acids. Adr1 expression is glucose-regulated in a strain-dependent fashion (5, 21) but is not Snf1 dependent (75). Its DNA binding domain (amino acids 76 to 160) (27, 50, 65, 66) and partially redundant transcription activation domains (TADI, amino acids 1 to 220; TADII, amino acids 263 to 359; TADIII, amino acids 420 to 462; and TADIV, amino acids 642 to 704) (15, 76) have been extensively characterized. Two inhibitory domains were found between amino acids 227 and 330 (15). The function, if any, of the C-terminal region (amino acids 705 to 1323) is unknown.

DNA binding of Adr1 is regulated posttranslationally and requires Snf1 when glucose is depleted (59, 76). Inactivation of the histone deacetylases Rpd3 and Hda1 allows Adr1 to bind promoters in repressing conditions, and the binding requires Snf1, suggesting that chromatin structure plays an inhibitory role and that Snf1 is required to overcome the inhibition (62, 70). A step in transcription after Adr1 binds is glucose repressed in the deacetylase mutant because the assembled PIC is inactive (62). It can be activated in repressing conditions by activating Snf1 and by mutant Adr1^c (62). Together, lack of deacetylase activity, activated Snf1, and activated Adr1 (Adr1^c) completely overcome glucose repression of gene expression (62).

In the presence of glucose, Adr1 is phosphorylated at two sites that appear to contribute to Adr1 inactivation. Phosphorylation of Ser98 in the DNA binding domain is dependent on the Pho80/Pho85 cyclin/Cdk pair. Loss of either Pho80 or Pho85 leads to a low level of constitutive, Adr1-dependent gene expression (42). Mutation of Ser98 to the phosphomimetic amino acid Asp inhibits DNA binding *in vitro* and *in vivo*, a result that is consistent with the nuclear magnetic resonance structure of an Adr1-DNA complex in which Ser98 makes close contacts with the phosphodiester backbone (7, 56). pSer98 is proposed to interfere with the backbone contacts and weaken DNA binding (42).

The second Adr1 phosphorylation site is Ser230 within the regulatory domain, amino acids 227 to 239. This domain was defined by 21 different mutations, *ADR1^c* alleles (constitutive *ADR1*), which caused constitutive, that is, glucose-insensitive *ADH2* expression (10, 12, 20–22). Because all 21 mutations occurred in this small region, it appears to be the only domain in Adr1 in which single point mutations can activate Adr1 in repressing conditions and cause constitutive *ADH2* expression. A Ser230-containing peptide is efficiently phosphorylated *in vitro* by protein kinase A (20) and by Ca^{2+}/calmodulin-dependent protein kinase (38), but neither of these kinases is essential for Ser230 phosphorylation *in vivo*, suggesting that it may be phosphorylated in the cell by multiple or redundant kinases (52). How Ser230 phosphorylation alters Adr1 activity is unknown. Adr1 phosphorylated on Ser230 can still bind DNA (53, 62), suggesting that a promoter-binding step in transcription may be inhibited by Ser230 phosphorylation. This is consistent with the ability of Adr1^c to activate the poised PIC in the deacetylase mutant.

In addition to causing constitutive *ADH2* expression, *ADR1^c* alleles alter the dependence of target gene expression on Cat8 and Oaf1/Pip2, two transcription factors that act in a combi-

natorial manner with Adr1 (4, 53). *ADR1^c* alleles also relieve the dependence on the histone acetyltransferase activity of the SAGA complex, although they remain dependent on the integrity of SAGA and on other coactivators (53). Surprisingly, Adr1 target genes that are strongly dependent on Snf1 for derepression are expressed in a Snf1-independent manner when Adr1^c is the activator (53). This observation may be mechanistically related to the Snf1-dependent dephosphorylation of pSer98 and pSer230 that occurs upon derepression (42). Whether the multiple phenotypes associated with *ADR1^c* alleles have a single origin or are caused by interactions with multiple target genes is unknown. Understanding the molecular basis of the Adr1^c phenotypes might provide an answer to this question, as well as yield new insight into transcription activation by Adr1.

Two models were proposed to explain the phenotype of the *ADR1^c* alleles (20), an intrasteric model in which the wild-type (WT) regulatory region masked an activation domain and a model invoking a repressor protein that bound to the regulatory region and inhibited transcription activation. We report that Bmh binds to the Adr1 regulatory domain and, thus, could be a direct inhibitor of Adr1 activity. When Bmh is active, the wild-type regulatory region inhibits a nearby activation domain. Substituting an Adr1^c regulatory region for the wild-type allele overcomes Bmh-mediated inhibition, suggesting that Bmh is unable to inactivate an activation domain associated with the Adr1^c allele. The experimental results support key elements of both models and suggest that Bmh may be the repressor invoked by Denis et al. (20) to explain the inhibitory role of the Adr1 regulatory domain.

MATERIALS AND METHODS

Yeast strains and growth of cultures. All strains used in this study are listed in Table 1 and were constructed by standard methods (57). Epitope tags and deletion mutations were introduced according to the methods of previously published work (17, 33, 43). Yeast cultures were grown in either yeast extract-peptone medium or in synthetic medium lacking the appropriate amino acid or uracil for plasmid selection. The repressed cultures contained 2 to 5% glucose, and the derepressed cultures contained 0.05% glucose. To maintain selection for plasmids containing *TRP1* (Trp$^+$) and/or *URA3*, the synthetic selective medium contained 0.2% Casamino Acids rather than the standard dropout solution.

Yeast two-hybrid assays. Yeast two-hybrid assays were performed in strain TYY304 essentially as described previously (41). The Gal4 activation domain (GAD)-*BMH1* plasmid (prey plasmid), pKD134, was from Dombek et al. (25). Fusions of genes encoding Adr1 proteins to the Gal4-DNA binding domain (GBD) (bait plasmids) were constructed in a *TRP1-CEN4* vector (pOBD2) expressing GBD-Adr1 from the strong, constitutive *ADH1* promoter (41, 67). All regions of Adr1, with the exception of the strong activation domain, TADIII, were included in the set of GBD-Adr1 fusions tested. Plasmids encoding GBD-Adr1 fusion proteins were made by gap repair in yeast (61). Briefly, pOBD2 was digested with PvuII and NcoI (67). PCR fragments representing various regions of *ADR1* were generated using forward and reverse primers that contained homology to the vector sequences flanking the polylinker region of pOBD2, as well as homology to *ADR1*. The NcoI-PvuII-digested pOBD2 DNA and a PCR fragment of the region of *ADR1* to be tested were used to transform TYY304 to Trp$^+$ prototrophy. Plasmid DNA from two or three Trp$^+$ transformants was rescued and sequenced to confirm that recombination had produced the correct in-frame gene fusion. Western analysis with an anti-GBD monoclonal antibody (RK5C1; Santa Cruz Biotechnology) was used to confirm the synthesis of a fusion protein of the correct size. The primers used are listed in Table S1 and the pOBD2-*ADR1* plasmids, called pGBDA1, pGBDA2, etc., in Table S2 at http://depts.washington.edu/younglab/PubSupData/MCB00715-10/.

Immunoprecipitation and Western blot analysis. Immunoprecipitations to concentrate samples for Western blot analyses and coimmunoprecipitations were carried out as described in reference 60 but using chromatin immunoprecipita-

TABLE 1. *Saccharomyces cerevisiae* strains used in the study

Strain	Genotype	Source
CKY19, aka[a] W303-1A	*MATa adr1Δ1::natmx ade2 can1-100 his3-11,15 leu2-13,112 trp1-1 ura3-1*	Yeast stock center
CKY13	CKY19 *adr1Δ1::natmx*	This study
TYY304, aka PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ*	41
	LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ	
NKY85[b]	*MATa trp1 ura3 leu2::(YIpADR1::LEU2)3 (ADR1-HA::kanmx)*	52, 59
YLL138	W303 *MATa bmh1Δ::HIS3*	46
YLL908	W303 *MATa bmh2Δ::kanmx*	46
YLL1087	W303 *MATa bmh2Δ::kanmx bmh1Δ::HIS3::bmh1-170*	46
YLL1083	W303 *MATa bmh2Δ::kanmx bmh1Δ::HIS3::bmh1-167*	46
YLL1120	W303 *MATa bmh2Δ::kanmx bmh1Δ::HIS3::bmh1-266*	46
SRY66	CKY13 *BMH1-myc3::HIS3*	This study
SRY69	YLL1087 *snf1Δ::natmx*	This study
CKY10	CKY19 *reg1Δ::natmx snf1Δ::kanmx*	This study
CKY11	CKY19 *reg1Δ::natmx*	This study
CKY17	CKY19 *snf1Δ::kanmx*	This study
EAY25	YLL1087 *adr1Δ::natmx*	This study
EAY29	YLL908 *adr1Δ::natmx*	This study

[a] aka, also known as.
[b] Has three copies of the pRS315-ADR1 (YEP-ADR1) plasmid integrated at the *leu2* locus. At least one copy is tagged with the HA epitope.

tion (ChIP) lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) with added protease and phosphatase inhibitors (Sigma), without DNase I treatment. Western blot analyses were performed according to the manufacturer's instructions for the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE), using 1:500 to 1:1,000 diluted polyclonal antihemagglutinin (anti-HA; Y-11) or monoclonal anti-myc (9E10) or anti-GBD as primary antibodies and Licor λ800 secondary antibodies. All antibodies were obtained from Santa Cruz Biotechnology.

Preparation of protein extracts from yeast cells. Protein extracts were prepared from 20 to 500 ml of yeast cells grown to an A_{600} of ~1. Cells were collected by centrifugation at 2,000 rpm for 5 min at 4°C in a Sorvall RC-3B plus centrifuge, washed once with 15% glycerol containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and resuspended in an equal volume of ChIP lysis buffer containing protease and phosphatase inhibitors (Sigma). The cells were broken with glass beads in a FastPrep machine with two disruption cycles of 45 s at a speed setting of 4.5 with an aliquot of PMSF added between cycles. After the second cycle, more protease and phosphatase inhibitors and one-half the original volume of ChIP lysis buffer were added. After the sample was vortexed briefly, the unbroken cells and debris were pelleted by centrifugation in a microcentrifuge at 13,000 rpm for 15 min. The clarified extract was used in subsequent experiments.

GST-Bmh pulldown assays. pGEX-3X-*BMH1* and pGEX-3X-*BMH2* were used to generate glutathione S-transferase (GST)–Bmh fusion proteins in *Escherichia coli* BL21(DE3) pLysS. The fusion proteins were bound to glutathione-Sepharose 4B beads as recommended by the manufacturer (GE Healthcare). Pulldown assays were performed with a 20- to 40-μl aliquot of freshly washed GST-Bmh beads (or GST beads) in 400 μl of phosphate-buffered saline (PBS) buffer containing protease and phosphatase inhibitors and 0.1% Tween 20. Clarified cell extracts were incubated with beads by mixing at 4°C for 2 h, and the beads were pelleted by centrifugation at 1,000 rpm for 1 min in a microcentrifuge. An aliquot of the supernatant (nonbound fraction) was removed and prepared for SDS-PAGE by adding an equal volume of 4× SDS-PAGE buffer and heating at 95°C for 5 min. The beads were washed four times with PBS containing 0.1% Tween 20, resuspended in 100 μl of 2× SDS-PAGE buffer, and heated at 95°C for 5 min. The beads were pelleted by centrifugation, and the supernatant was removed for SDS-PAGE analysis. This fraction is referred to as the bound or pellet fraction.

mRNA isolation and qRT-PCR. mRNA was isolated from strains grown in either repressing or derepressing medium using the hot phenol method described by Collart and Oliviero (14). Residual DNA in the RNA preparation was reduced by treatment with DNase I (Ambion) following the manufacturer's recommendations. cDNA synthesis was performed with SuperScript III (Invitrogen) following the manufacturer's protocol. Quantitative real-time reverse transcription (RT)-PCR (qRT-PCR) for measuring mRNA levels was performed using a 1:300 dilution of the cDNA. A standard curve was generated from *ACT1* primers and used to quantify all of the RNA levels. Samples were prepared from biological triplicates and analyzed in duplicate.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Guarente (32) after growing the cells at 30°C in selective medium containing 2 to 5% glucose to an A_{600} of ~1. The reported values in Miller units are the averages of the results for three to five transformants.

RESULTS

Bmh inhibits WT Adr1 activity in W303. Previous studies of the role of Bmh in glucose repression in *S. cerevisiae* used the Σ1278a strain (25, 40), which is viable in the absence of any Bmh function, unlike common laboratory strains (69). Because the Σ1278a strain might have a suppressor that is responsible for overcoming glucose repression, we tested three *bmh2Δ bmh1-ts* mutants isolated from the common laboratory strain W303 (46). All three mutants displayed constitutive *ADH2* expression under high-glucose conditions (Fig. 1). In addition, reduced Bmh activity was associated with higher levels of expression of Adr1 target genes in derepressing conditions. Thus, as in the Σ1278a strain, there was a high level of glucose-resistant *ADH2* expression in the absence of Bmh function. Because the overexpression of Adr1 also causes constitutive *ADH2* expression and hyperderepression (5, 18), we measured *ADR1* expression and found that it was lower in the *bmh1-ts* mutants (Fig. 1). Therefore, constitutive *ADH2* expression and enhanced derepression are not due to *ADR1* overexpression. In conclusion, reduced Bmh activity leads to higher levels of Adr1-dependent gene expression in both the presence and absence of glucose, suggesting that Bmh might inhibit Adr1 activity in both conditions.

Strain YLL1087 (*bmh2Δ bmh1-170*) produced a higher level of constitutive *ADH2* expression than the other *bmh1-ts* strains, so it was used for subsequent studies. We showed that constitutive *ADH2* expression and derepression of Adr1-dependent genes was Adr1 dependent in the *bmh1-ts bmh2Δ* strain (25; data not shown) and also showed that Adr1-dependent gene expression and Adr1 promoter binding were dependent on Snf1 in this background (see Fig. S1A and S1B at http://depts.washington.edu/younglab/PubSupData/MCB00715-10/). Thus, Adr1-dependent gene expression is regulated by the
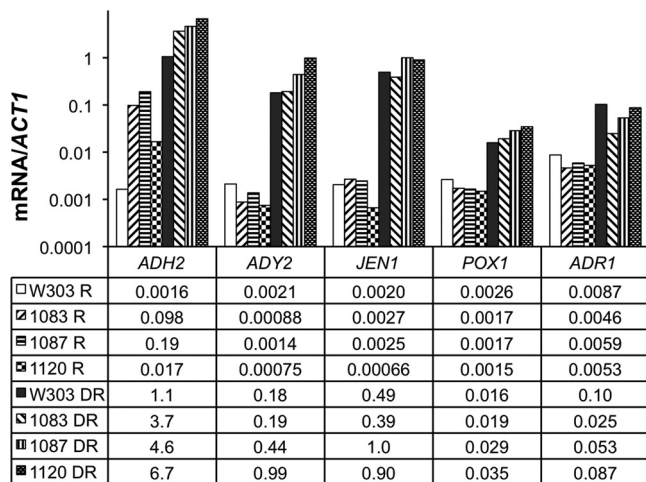
FIG. 1. Adr1-dependent gene expression in *bmh1-ts* strains. W303-1A is WT for both *BMH* genes; the other strains are *bmh1*Δ::*HIS3 bmh2*Δ::*kanmx4* with integrated *bmh1-ts* alleles: *bmh1-167*::*LEU2* (YLL1083), *bmh1-170*::*LEU2* (YLL1087), and *bmh1-266*::*LEU2* (YLL1120) (Table 1). R, repressing growth conditions (3% glucose); DR, derepressing growth conditions (0.05% glucose).

Snf1 pathway in the absence of a functional *BMH* gene as it is in a *BMH* wild-type strain. We also showed that Adr1-dependent gene expression in the absence of Bmh activity was not enhanced by Adr1[c] (see Fig. S1C at the URL above), suggesting that both mutations alter the same pathway of glucose repression.

Bmh interacts directly with Adr1. We hypothesized that a Reg1-independent role of Bmh in the repression of Adr1-dependent genes (25) might be as a direct inhibitor of Adr1 because a global analysis identified Adr1 as one of more than 60 proteins that copurified with Bmh1 (37). The results of our coimmunoprecipitation experiments show that immunoprecipitation of Bmh1-Myc from yeast cell extracts also pulls down Adr1-HA (Fig. 2A). Further evidence for a physical interaction was obtained from glutathione *S*-transferase (GST) pulldown assays using purified recombinant GST-Bmh1 and yeast extracts prepared from a strain overexpressing Adr1-HA. Adr1-HA was observed in the pulldown fraction with GST-Bmh1 but not with GST alone (Fig. 2B), showing that the interaction is specific for the Bmh portion of the fusion protein.

Role of Ser230 phosphorylation in Adr1-Bmh interaction. Adr1 phosphorylated on Ser230 (pSer230-Adr1) might be preferentially associated with GST-Bmh1, because activation by Adr1-S230A was insensitive to the loss of Bmh function (see Fig. S1C at the URL above) and 14-3-3 proteins bind phosphorylated substrates. We performed GST-Bmh1 pulldown assays using cell extracts prepared from a strain overexpressing Adr1-HA and then probed a Western blot containing bound and extract fractions with anti-pSer230 antibodies to detect pSer230-Adr1 (52) and with anti-HA antibodies to detect total Adr1. The pSer230 signal, normalized to the HA signal, in the bound fraction was 12-fold higher than in the whole-cell extract (Fig. 3). This result suggests that Bmh preferentially interacts with pSer230-Adr1.
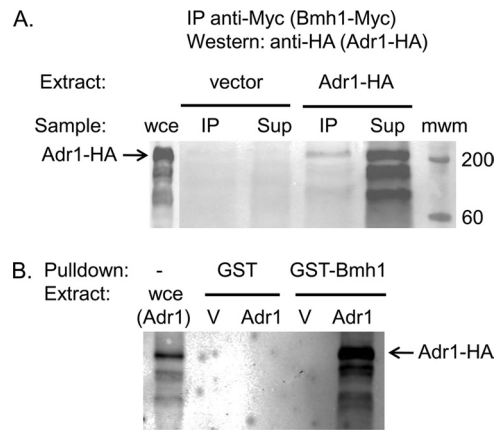

FIG. 2. Interaction between Bmh1 and Adr1. (A) Adr1 coimmunoprecipitates with Bmh1. Bmh1-Myc was immunoprecipitated with antibodies to the Myc tag from the SRY66 strain with either the Adr1-HA expression plasmid (pKD17HA) or an empty plasmid (pKD8). Both the immunoprecipitated (IP) and nonbound supernatant (Sup) fractions were analyzed by Western blotting using antibodies against the HA tag. The position of Adr1-HA is shown in the first lane, containing the whole-cell extract (wce). The molecular weight markers (mwm; in thousands) are shown in the last lane. (B) Recombinant GST-Bmh1 interacts with Adr1-HA from yeast extracts. Glutathione-Sepharose beads bound to either recombinant GST-Bmh1 or GST were used in pulldown assays with extracts from an *adr1*Δ strain (CKY13) with either the Adr1-HA expression plasmid (pKD17HA) or an empty plasmid (pKD8). The bound fractions were analyzed by Western blotting using antibodies against the HA tag. The whole-cell extract (wce) for the Adr1-HA-expressing strain is shown in the first lane. The position of Adr1-HA is shown on the right. Equal amounts of GST-Bmh1 and GST were bound to the beads, as detected by Coomassie brilliant blue staining (not shown).

Bmh can interact with Adr1 present in extracts from repressed and derepressed cells. To test if the interaction of Adr1 with Bmh is glucose regulated, pulldown experiments with GST-Bmh were performed using extracts from cells grown in repressing and derepressing conditions. A strain containing four integrated copies of a plasmid expressing *ADR1* from its own promoter was used to amplify the signal (59). Adr1-HA was pulled down by GST-Bmh1 from both types of extracts

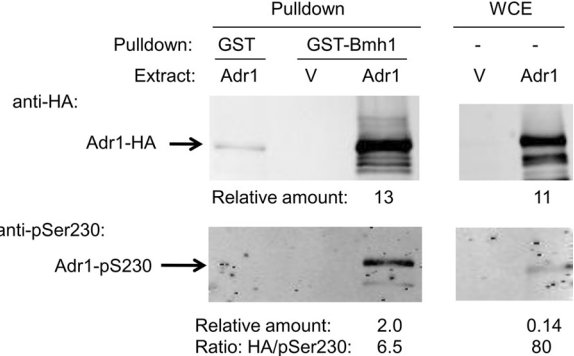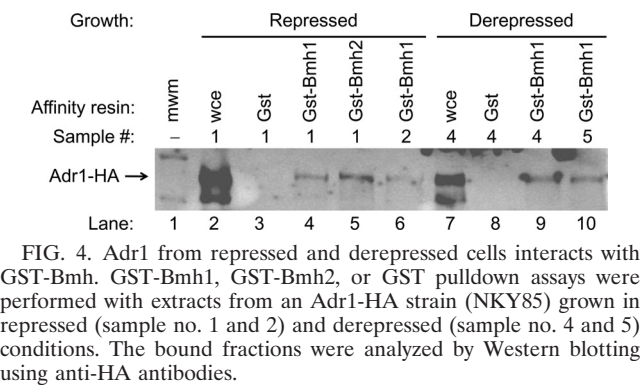FIG. 3. GST-Bmh1 preferentially interacts with Adr1 phosphorylated at Ser230. GST-Bmh1 pulldown assays were performed as described in the Fig. 2B legend. The bound fractions were analyzed by Western blotting using anti-HA and anti-pSer230 antibodies. The positions of Adr1-HA and Adr1-pS230 are shown on the left. The relative amounts of Adr1 were quantitated and are shown below the relevant lanes.

FIG. 4. Adr1 from repressed and derepressed cells interacts with GST-Bmh. GST-Bmh1, GST-Bmh2, or GST pulldown assays were performed with extracts from an Adr1-HA strain (NKY85) grown in repressed (sample no. 1 and 2) and derepressed (sample no. 4 and 5) conditions. The bound fractions were analyzed by Western blotting using anti-HA antibodies.

(Fig. 4), suggesting that Adr1 might interact *in vivo* with Bmh1 in both repressing and derepressing growth conditions. A similar result was obtained using GST-Bmh2 (Fig. 4, lane 5), indicating that Adr1 can interact directly with both Bmh1 and Bmh2. Because the interaction appears to require phosphorylation (Fig. 3), these results suggest that Adr1 is phosphorylated on Ser230 in both repressing and derepressing conditions. Indeed, using the same multicopy *ADR1* strain, it was

shown that Adr1 phosphorylated on Ser230 decreased but was still present after derepression (52).

Bmh interacts with the regulatory region of Adr1. Two-hybrid assays using GBD-Adr1 and GAD-Bmh1 fusion proteins confirmed the physical interaction between Bmh1 and Adr1 and identified the region of Adr1 involved in the interaction. Figure 5 shows the Adr1 fragments that were fused to GBD. The results of the two-hybrid assays suggested that Bmh1 interacts with the regulatory region of Adr1, between amino acids 220 and 304 (see Fig. S2A and B at http://depts .washington.edu/younglab/PubSupData/MCB00715-10/).

GST-Bmh1 pulldown assays with yeast extracts prepared from strains expressing the GBD-Adr1 fusion proteins confirmed the site of interaction. GST-Bmh1 interacted with the fusion protein containing the region encompassing Adr1 amino acids 148 to 304 [GBD-Adr1(148–304)] but not with GBD-Adr1(20–220), GBD-Adr1(468–1323), or GBD alone (Fig. 6). Together, these results suggest that the region of Adr1 between amino acids 220 and 304, which includes the regulatory region, interacts with Bmh.

Bmh binds in the region defined by Adr1 amino acids 215 to 260. The region of Adr1 that interacts with Bmh contains three
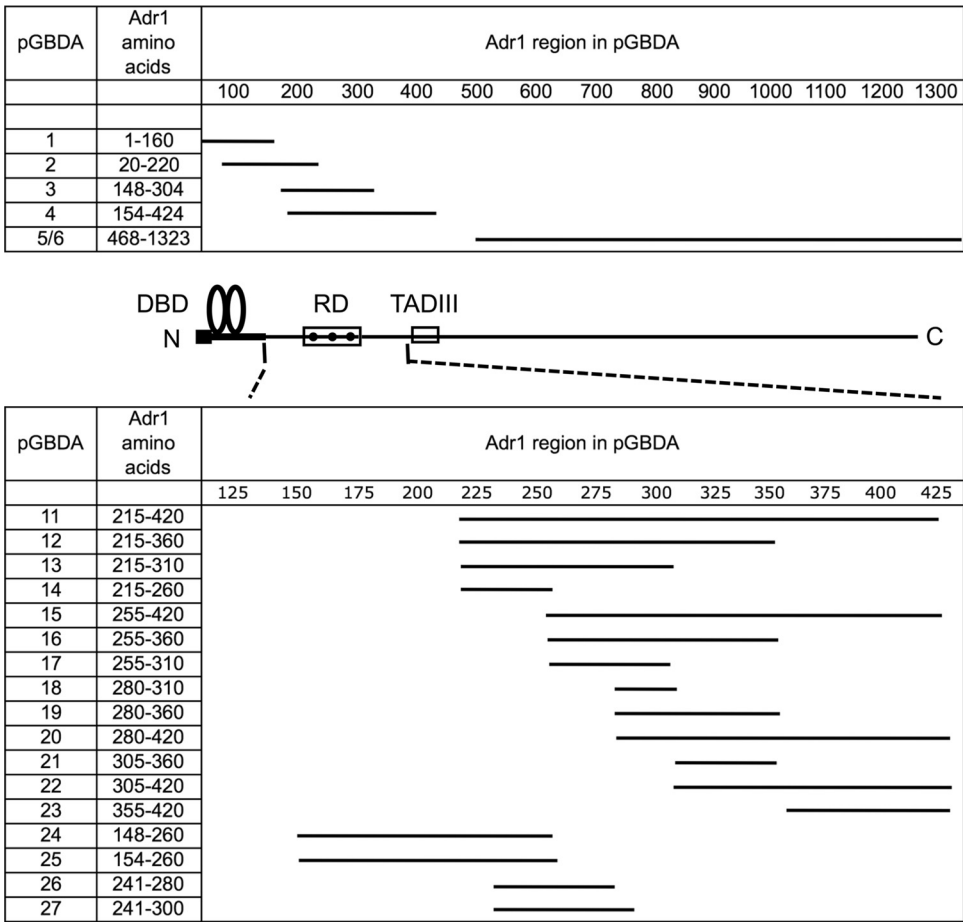

FIG. 5. Gal4-Adr1 fusion proteins. The figure depicts a schematic of the Adr1 protein and the Adr1 fragments found in the two sets of Gal4-Adr1 fusion protein-expressing plasmids (pGBDA variants). DBD, Adr1 DNA binding domain, amino acids 76 to 160 (27, 66); RD, regulatory domain, residues 220 to 330 (15); TADIII, transcription activation domain III, residues 420 to 462 (15, 76). The dots in the RD box indicate the approximate locations of Ser230, Ser258, and Ser301 within putative 14-3-3 binding motifs.
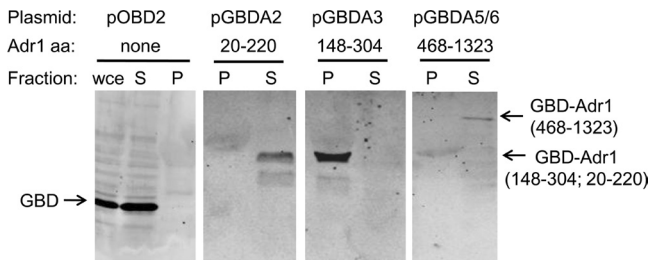
FIG. 6. GST-Bmh1 pulldown assays identify the regulatory domain of Adr1 as the site of interaction with Bmh. GST-Bmh1 pulldown assays were performed using extracts from strain TYY304 with one of the pGBDA plasmids or the control GBD plasmid (pOBD2). The bound or pellet (P) and nonbound or supernatant (S) fractions were analyzed by Western blotting using antibodies against GBD. The positions of the GBD-Adr1 fusion proteins are shown to the right, and GBD to the left, in the whole-cell extract (wce). aa, amino acids.

close matches to a 14-3-3 consensus motif, $RRAS_{230}FA$, $RVKFS_{258}TP$, and $RSDS_{301}SG$. To further refine the location of the Bmh binding site and determine the importance of the 14-3-3 motifs, we constructed a series of plasmids (pGBDA11 to pGBDA27) (Fig. 5, bottom) encoding GBD-Adr1 fusion proteins with various N- and C-terminal endpoints between Adr1 amino acids 148 and 420. Yeast two-hybrid assays with these GBD-Adr1 fusion proteins demonstrated that only those fusion proteins that contained the amino acid 215 to 260 region interacted with GAD-Bmh1 (Fig. 7A; also see Fig. S3 at http://depts .washington.edu/younglab/PubSupData/MCB00715-10/). The fusion proteins that lacked this region had such high activity in the control strain lacking GAD-Bmh1 that any two-hybrid interaction was masked.

The problem of high two-hybrid background signal was circumvented by using GST-Bmh pulldown assays with the GBD-Adr1 fusion proteins to identify the site of Adr1-Bmh interaction. Fusion proteins containing the region encompassing Adr1 amino acids 215 to 260 bound to GST-Bmh1, and those lacking this region, including a region spanning amino acids 240 to 310, showed no binding (Fig. 7B and data not shown). This excludes the Ser258 and Ser301 motifs as Bmh binding sites and indicates that there is a Bmh binding site between Adr1 amino acids 215 and 260. This region includes all of the *ADR1^c* alleles located between amino acids 227 and 239 and suggests that the *ADR1^c* phenotypes may be associated with altered Bmh binding.

The regulatory domain contains a cryptic activation region that is unmasked by *ADR1^c* alleles. In the two-hybrid assays, we noticed that many of the GBD-Adr1 fusions containing amino acids 255 to 360 were active with pGAD alone but only if they lacked the region between Adr1 amino acids 215 and 260 (Fig. 7A; also see Fig. S3 at http://depts.washington.edu /younglab/PubSupData/MCB00715-10/). These results suggest that there is a cryptic activating region, which we will refer to as a cryptic activation domain (AD), between amino acids 255 and 360 that is inhibited by the Bmh binding region, comprised of amino acids 215 to 260. The AD in this region could be TADII, which was detected as an AD when a similar region was overexpressed as a LexA-Adr1 fusion protein (15).

Surprisingly, similar GBD-Adr1 fusion proteins that contained the cryptic AD and had an S230A mutation (GBD-

Adr1:S230A) within the Bmh binding region were active. Table 2 shows the results of β-galactosidase reporter assays with the plasmids expressing GBD-Adr1(148–304) and GBD-Adr1(154–424) with either the S230A (*ADR1^c*) or WT allele. The fusion proteins containing the S230A allele stimulated much higher reporter expression than those containing the WT allele (Table 2), indicating that Adr1 contains an AD between

Gal4-Adr1 fusion plasmid		β-galactosidase activity		Growth [1]			
				Prey plasmid			
pGBDA	Adr1 aa	pGAD	pGAD-BMH	pGAD		pGAD-BMH	
				His	Ade	His	Ade
11	215-420	3.5	7.5	++	+	++	++
12	215-360	3.6	5.8	++	--	++	+
13	215-310	12	31	++	--	++	+
14	215-260	2.5	4.2	--	--	+	--
15	255-420	140	82	++	++	++	++
16	255-360	76	48	++	++	++	++
17	255-310	110	90	++	++	++	++
18	280-310	32	20	++	--	++	--
19	280-360	49	10	++	++	++	++
20	280-420	110	37	++	++	++	++
21	305-360	7.8	2.6	++	--	++	--
22	305-420	24	8.8	++	+	++	+
23	355-420	6.0	4.4	+	--	+	--
24	148-260	2.2	1.3	--	--	--	--
25	154-260	1.7	1.1	--	--	--	--

[1] ++, growth of all three spots similar to control plates ; --, no growth.
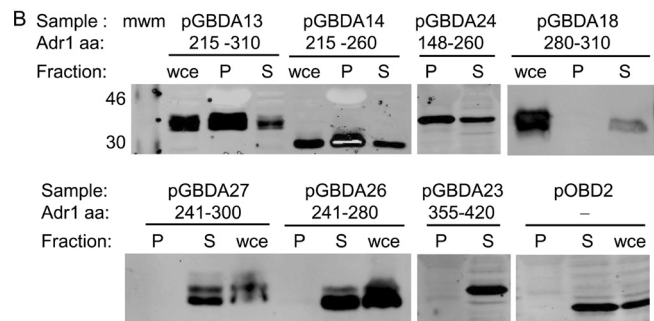

FIG. 7. Refining the site of Adr1-Bmh interaction using pGBDA11 to pGBDA27. (A) Summary of the results of two-hybrid assays, including growth tests and β-galactosidase activity assays, for the fusion proteins in plasmids pGBDA11 to -25 in the strain TYY304. (B) GST-Bmh1 pulldown assays were performed as described in the Fig. 2 legend. mwm, molecular weight markers, in thousands.

TABLE 2. GBD-Adr1:S230A is active without a two-hybrid partner

Bait[b]	Prey	β-Galactosidase activity (avg ± 1 SD)[a]
GBD-Adr1(148–304)	GAD	1.9 ± 0.7
GBD-Adr1(148–304)	GAD-Bmh1	8.6 ± 1.8
GBD-Adr1(148–304:S230A)	GAD	19 ± 3.9
GBD-Adr1(148–304:S230A)	GAD-Bmh1	15 ± 3.0
GBD-Adr1(154–424)	GAD	0.35 ± 0.05
GBD-Adr1(154–424)	GAD-Bmh1	7.0 ± 0.8
GBD-Adr1(154–424:S230A)	GAD	3.6 ± 0.4
GBD-Adr1(154–424:S230A)	GAD-Bmh1	7.6 ± 1.2

[a] Strain TYY304 (Table 1) was transformed with plasmids expressing the indicated fusion proteins. β-Galactosidase activity was measured as described in Materials and Methods. The values are the averages of the results for three to five transformants ± one standard deviation.

[b] The bait and prey plasmids are listed in Table S2 at http://depts.washington.edu /younglab/PubSupData/MCB00715-10/.

TABLE 3. Bmh represses GBD-Adr1 through the regulatory domain

Plasmid[e]	GBD-Adr1 fusion protein expressed	β-Galactosidase activity (avg ± 1 SD)[a] in strain with genotype:		
		BMH1[b]	bmh1-170[c]	bmh1Δ BMH2[d]
pGBDA2	GBD-Adr1(20–220)	0.2 ± 0.13	0.8 ± 0.5	NM
pGBDA3	GBD-Adr1(148–304)	0.22 ± 0.04	17 ± 2.7	0.7 ± 0.2
pGBDA3SA	GBD-Adr1(148–304:S230A)	8.4 ± 3.1	13 ± 1.9	28 ± 1.7
pGBDA4	GBD-Adr1(154–424)	0.6 ± 0.2	52 ± 6.6	NM
pGBDA4SA	GBD-Adr1(154–424:S230A)	42 ± 2.8	36 ± 0.8	NM

[a] β-Galactosidase (expressed from reporter plasmid pH218′) activity was measured as described in Materials and Methods. The values are the averages of the results for three to six transformants ± one standard deviation. NM, not measured.
[b] Strain YLL908 *BMH1 bmh2Δ* (Table 1).
[c] Strain YLL1087 *bmh1Δ bmh2Δ bmh1-170* (Table 1).
[d] Strain YLL138 *BMH2 bmh1Δ* (Table 1).
[e] The plasmids are listed in Table S2 at http://depts.washington.edu/younglab/PubSupData/MCB00715-10/.

amino acids 148 and 304 that is inhibited in the presence of Ser230 but not in the presence of Ala230. Other GBD-*ADR1*[c] alleles (R228K, S230D, and Δ3 [lacking amino acids 226 to 233]) behaved similarly (data not shown). Because most *ADR1*[c] fusion proteins differ from the WT by a single amino acid change in their regulatory region, the most plausible explanation for their different activities is that the cryptic AD is inhibited in the WT protein and the inhibition is relieved by the *ADR1*[c] alleles.

Bmh inhibits the cryptic activating region in the regulatory domain. One possible explanation for the high activity of the GBD-Adr1[c] proteins is that Bmh binding is inhibited, thus unveiling activation domains. If Bmh inhibits the function of the cryptic AD in the regulatory region, the WT GBD-Adr1 fusion proteins might be active in a strain lacking a functional *BMH* allele.

We tested this possibility by transforming the *bmh1-ts* strain (YLL1087) and its parent (YLL908) with plasmids expressing WT Adr1 and Adr1[c] as GBD fusion proteins. WT GBD-Adr1(148–304)- and GBD-Adr1(154–424)-expressing plasmids were active in the *bmh1-ts* strain but inactive in the strain containing WT *BMH1* (Table 3). They were also inactive in a strain with WT *BMH2* and no *BMH1* (data not shown). The activities of the GBD-Adr1[c] activators, GBD-Adr1(148–304: S230A) and GBD-Adr1(154–424:S230A) were equivalent in the WT and mutant strains, suggesting that Bmh and Adr1[c] affect the same or dependent steps in transcription activation. GBD-Adr1(20–220), which lacks the regulatory domain, was inactive in both strains. We confirmed these results using strains with two other *bmh1-ts* alleles, *bmh1-167* (YLL1083) and *bmh1-266* (YLL1120) (data not shown). In summary, the regulatory region of Adr1 appears to contain a Bmh-inhibited activating region that is separable from the Bmh binding site.

Bmh inhibition requires the amino acid 215 to 260 region of Adr1. To identify more precisely the region required for Bmh-dependent inhibition, we introduced pGBDA11 to -27 into a WT *BMH1* strain (YLL908) and the *bmh1-170-ts* mutant (YLL1087), each containing a *GAL4*-dependent *lacZ* reporter, and performed β-galactosidase activity assays (Fig. 8). The fusion proteins containing Adr1 amino acids 215 to 420, 215 to 360, and 215 to 310 were inactive in the *BMH1* WT strain, whereas most of those lacking the amino acid 215 to 260 region were active. Thus, the Bmh binding region, amino acids 215 to 260, appears to inhibit the activity of the cryptic AD.

The results obtained with the *bmh1-170* strain were strikingly different. The plasmids expressing GBD fusion proteins containing Adr1 amino acids 215 to 420, 215 to 360, and 215 to 310 were active in the *bmh1-ts* mutant, consistent with Bmh-mediated inhibition of an AD that is present in these fusion proteins. The fusion proteins containing only the Bmh-interacting region of Adr1 [GBD-Adr1(215–260), GBD-Adr1(148–260), and GBD-Adr1(154–260)] were inactive in both strains. These results localize the amino acids necessary and sufficient for inhibition to the Bmh binding region, amino acids 215 to 260, and confirm that the cryptic AD is separable from the inhibitory region. In addition, they demonstrate that deleting the Bmh binding region from the regulatory region is equivalent to inactivating Bmh.

Bmh inhibition of GBD-Adr1 fusion proteins acts on endogenous *GAL* targets. To determine if Bmh-mediated inhibition is dependent upon promoter context, we examined whether Bmh can inhibit endogenous *GAL4*-regulated promoters when GBD-Adr1 activators are present. *GAL1*, *GAL7*, and *GAL10* mRNA levels in the WT *BMH1* strain and in the *bmh1-170* mutant were measured by qRT-PCR. RNA levels from the *GAL* genes were markedly higher in the *bmh1-ts* mutant strain
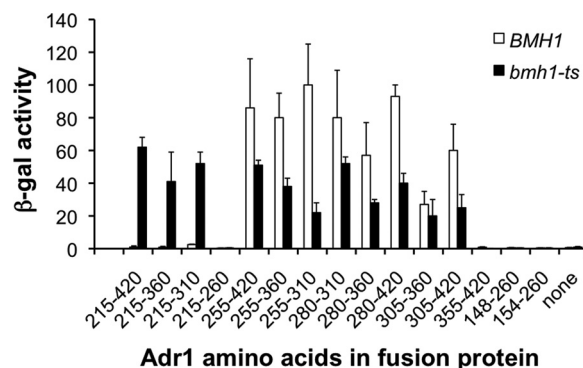
FIG. 8. Bmh inhibits GBD-Adr1 through the Bmh binding domain, amino acids 215 to 260. Strains YLL908 (*BMH1 bmh2Δ*) and YLL1087 (*bmh1Δ bmh2Δ bmh1-170*) were transformed with a plasmid expressing a *CYC1-lacZ* fusion protein under the control of the *GAL10* promoter (pHZ18′) and plasmids expressing various GBD-Adr1 fusion proteins (pGBDA variants). The level of *lacZ* expression was assessed using a β-galactosidase activity assay and plotted in Miller units ± 1 standard deviation.

FIG. 9. Bmh inhibits GBD-Adr1 transcriptional activity at endogenous GAL promoters. mRNA was purified from strains YLL908 (bmh2Δ) and YLL1087 (bmh1Δ bmh2Δ bmh1-170) with pOBD2, pGBDA4, or pGBDA4SA. Quantitative RT-PCR 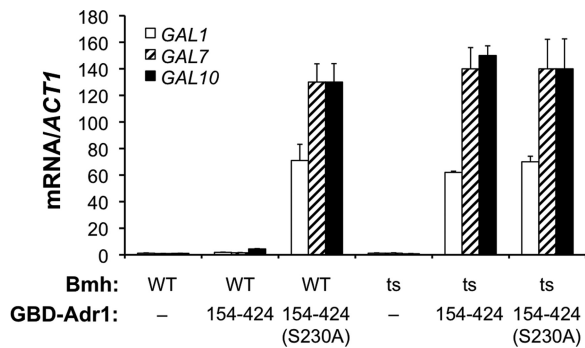was performed to determine the levels of GAL1, GAL7, and GAL10 mRNA. The levels of GAL mRNA were normalized to the level of ACT1 mRNA in each sample.

than in the WT BMH1 strain when GBD-Adr1(154–424) was present (Fig. 9). In contrast, equivalent levels of RNA were present in the BMH1 WT and bmh1-170 mutant when GBD-Adr1(S230A) was the activator, and these levels were equivalent to those with the WT activator in the bmh1-170 mutant. GAL mRNA levels in strains with the vector control (pOBD2) were very low in both strains, indicating the absence of activation by endogenous Gal4. Therefore, Bmh-mediated inhibition of GBD-Adr1 requires a WT Ser230 allele and inhibition is not promoter context dependent but is activator dependent.

Activation of GBD-Adr1 in the absence of Bmh-mediated inhibition is independent of Snf1. Adr1[c] bypasses Bmh-mediated inhibition (Tables 2 and 3; also see Fig. S1C at http://depts .washington.edu/younglab/PubSupData/MCB00715-10/), suggesting that Snf1-dependent dephosphorylation of pSer230 (52) might prevent the Bmh-mediated inhibition of Adr1 activity. If this idea is correct, the Gal4-Adr1 fusion proteins might be active independent of Snf1 in a bmh1-ts strain. We tested this possibility by determining the Snf1 dependence of both WT and Adr1[c]-containing fusion proteins in a bmh1-ts strain and found that the fusion proteins were indeed Snf1 independent in the absence of Bmh function (Table 4). Although the activity was about 2-fold higher in the bmh1-170 snf1Δ double mutant than in the bmh1-170 SNF1 parent, this was true for all three GBD proteins, GBD alone, GBD-Adr1 WT, and GBD-Adr1[c], and could be an indirect effect due to

the slower growth rate of the double mutant. Thus, the results suggest that activation does not require Snf1 if the regulatory domain cannot bind Bmh either because Bmh is absent or because it is refractory to Bmh-mediated inhibition due to an ADR1[c] mutation.

If Bmh-mediated inhibition requires phosphorylated Ser230 and if Snf1 promotes dephosphorylation of GBD-Adr1 as it does in WT Adr1 (52), then activating Snf1 might enhance GBD fusion protein-dependent expression in a WT BMH1 strain. To test this possibility, we used a strain in which REG1 had been deleted (CKY11). The results in the last two columns of Table 4 show that deleting REG1 in a WT BMH1 strain enhanced expression about 10-fold and that the increase was Snf1-dependent. In the WT REG1 BMH1 strain, the expression was very low when WT GBD-Adr1 was the activator and high when the S230A allele was the activator. GBD-Adr1 with the S230A allele was also insensitive to the REG1 and SNF1 genotypes, as predicted if S230 phosphorylation is the only target of Snf1 in the fusion protein. The enhanced activation in the reg1Δ strain demonstrates that the activity of the GBD-Adr1 fusion protein has not bypassed the requirement for Snf1.

The Adr1 regulatory region contains two conserved Bmh-related sequences. An independent assessment of the importance of the regulatory region to Adr1 function was made by determining its evolutionary conservation. The Gene Order Browser (31) was used to identify ADR1 orthologs in the genomes of other Ascomycetes. The DNA binding domain of Adr1 is a close match to that of four other zinc finger proteins in S. cerevisiae, RSF2/ZMS1, YML081C, YGR067C, and YPR022C (6). RSF2 and YML081C are ohnologs, paralogs created by the whole-genome duplication (WGD), and appear to be true ADR1 homologs based on further sequence analysis. Adr1 has no ohnolog. Recovering syntenic orthologs of Adr1 and Rsf2 from the Gene Order Browser yielded 11 and 13 open reading frames (ORFs), respectively. We expanded our comparison of Adr1 to include Mxr1 in Pichia pastoris because it was reported to be an ADR1 ortholog based on its similar DNA binding domain, DNA binding properties, and gene activation profile (45). All potential Adr1 and Rsf2 orthologs show a high degree of sequence conservation across the entire ORF, varying from 80% sequence identity for the Adr1 homolog from Saccharomyces bayanus to 22% sequence identity for Mxr1 (see Table S3 at http://depts.washington.edu /younglab/PubSupData/MCB00715-10/).

TABLE 4. Activation by GBD-Adr1(148–304) is Snf1 independent in the absence of Bmh

Plasmid[b]	β-Galactosidase activity (avg ± SD)[a] in strain with genotype[c]:					
	BMH1 SNF1	BMH1 snf1Δ	bmh1-170 SNF1	bmh1-170 snf1Δ	reg1Δ SNF1	reg1Δ snf1Δ
pOBD2	0.5 ± 0.01	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.09 ± 0.01	0.11 ± 0.02
pGBDA3	0.5 ± 0.1	0.6 ± 0.2	31 ± 9.0	64 ± 8.0	1.3 ± 0.55	0.17 ± 0.1
pGBDA3SA	92 ± 4.0	100 ± 10	33 ± 10	60 ± 12	24 ± 1.0	32 ± 5.0

[a] β-Galactosidase activity was measured as described in Materials and Methods. The results are the averages and standard deviations from three to six transformants assayed in two to three experiments.
[b] pOBD2 is the GBD vector with no ADR1 insert; pGBDA3 and pGBDA3SA express GBD-Adr1(148–304) with the WT or S230A allele, respectively. The plasmids are described in Table S2 at http://depts.washington.edu/younglab/PubSupData/MCB00715-10/.
[c] BMH1 SNF1, strain CKY19; BMH1 snf1Δ, strain CKY17; bmh1-170 SNF1, strain YLL1087; bmh1-170 snf1Δ, strain SRY69; reg1Δ SNF1, strain CKY11; reg1Δ snf1Δ, strain CKY10 (Table 1). All strains carry the GAL10-lacZ reporter pHZ18′ (see Table S2 at the URL in footnote b).

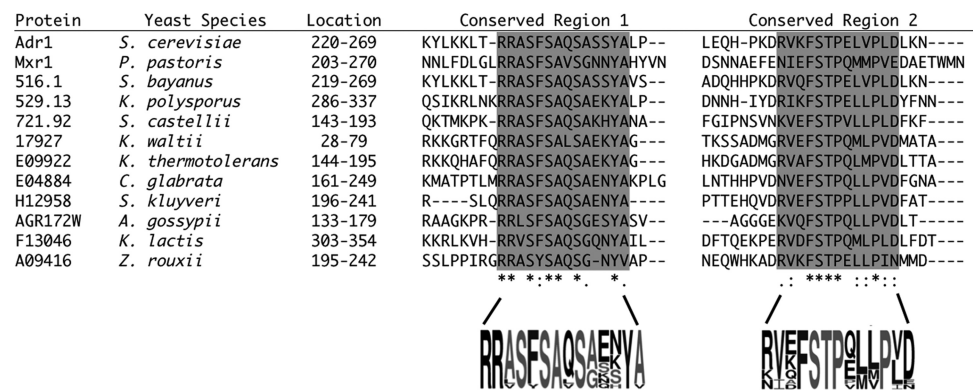| Protein	Yeast Species	Location	Conserved Region 1	Conserved Region 2
Adr1	*S. cerevisiae*	220-269	KYLKKLT-RRASFSAQSASSYALP--	LEQH-PKDRVKFSTPELVPLDLKN----
Mxr1	*P. pastoris*	203-270	NNLFDLGLRRASFSAVSGNNYAHYVN	DSNNAEFENIEFSTPQMMPVEDAETWMN
516.1	*S. bayanus*	219-269	KYLKKLT-RRASFSAQSASSYAVS--	ADQHHPKDRVQFSTPELVPLDLKN----
529.13	*K. polysporus*	286-337	QSIKRLNKRRASFSAQSAEKYALP--	DNNH-IYDRIKFSTPELLPLDYFNN---
721.92	*S. castellii*	143-193	QKTMKPK-RRASFSAQSAKHYANA--	FGIPNSVNKVEFSTPVLLPLDFKF----
17927	*K. waltii*	28-79	RKKGRTFQRRASFSALSAEKYAG---	TKSSADMGRVEFSTPQMLPVDMATA---
E09922	*K. thermotolerans*	144-195	RKKQHAFQRRASFSAQSAEKYAG---	HKDGADMGRVAFSTPQLMPVDLTTA---
E04884	*C. glabrata*	161-249	KMATPTLMRRASFSAQSAENYAKPLG	LNTHHPVDNVEFSTPQLLPVDFGNA---
H12958	*S. kluyveri*	196-241	R----SLQRRASFSAQSAENYA----	PTTEHQVDRVEFSTPQLMPVDDFAT---
AGR172W	*A. gossypii*	133-179	RAAGKPR-RRLSFSAQSGESYASV--	---AGGGEKVQFSTPQLLPVDLT-----
F13046	*K. lactis*	303-354	KKRLKVH-RRVSFSAQSGQNYAIL--	DFTQEKPERVDFSTPQMLPLDLFDT---
A09416	*Z. rouxii*	195-242	SSLPPIRGRRASYSAQSG-NYVAP--	NEQWHKADRVKFSTPELLPINMMD----

FIG. 10. Conserved motifs in the regulatory domain of Adr1. *ADR1* orthologs were recovered from the Gene Order Browser (31) and aligned using the ClustalW 2.0 World Wide Web service at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/) (64, 44). The *MXR1* sequence was obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein/88866603) (45). The amino acid consensus logos were created using WebLogo 3.0 (http://weblogo.threeplusone.com/create.cgi) (16). The yeast species are *Saccharomyces cerevisiae*, *Pichia pastoris*, *Saccharomyces bayanus*, *Kluyveromyces polysporus*, *Saccharomyces castelli*, *Kluyveromyces waltii*, *Kluyveromyces thermotolerans*, *Candida glabrata*, *Saccharomyces kluyveri*, *Ashbya gossypii*, *Kluyveromyces lactis*, and *Zygosaccharomyces rouxii*.

All of the Adr1 orthologs, including Mxr1, possess regions corresponding to Adr1 amino acids 227 to 240 and 254 to 266 that are highly conserved in both sequence and location (Fig. 10). The first of these regions coincides perfectly with the Bmh binding site and the location of the *ADR1*^{c} alleles. It is similar to 14-3-3 motifs (73) but lacks the conserved Pro at the +2 position with respect to the phosphorylated Ser230. Conserved region 1 differs in a consistent manner between the Adr1 and Rsf2 orthologs (see Fig. S4 at the URL above), suggesting the duplication of an ancestral *ADR1/RSF2* gene that had a similar regulatory region prior to the WGD.

Conserved region 2 is more similar to a 14-3-3 binding motif than is conserved region 1 and varies in a consistent manner between the Adr1 and Rsf2 orthologs. The highly conserved nature of these sequences, their similar separation and location, and their presence in homologous proteins, all of which are putative or demonstrated (Adr1, Rsf2, and Mxr1) transcription factors, strongly argue that they have an important function. For conserved region 1, this function is related to Bmh binding. The role of conserved region 2 is unknown. We did not detect Bmh binding to a GBD-Adr1 fusion protein containing this region (Fig. 7B). There was essentially no conservation detected in the region containing the cryptic AD, amino acids 266 to 360.

DISCUSSION

The studies reported here demonstrate that Bmh binds to the Adr1 regulatory region, defined by *ADR1*^{c} alleles (20) and by the deletion studies of Cook et al. (15) as lying between Adr1 amino acids 227 and 330. Specifically, we show that Bmh binds between amino acids 215 and 260, the site of all of the *ADR1*^{c} mutations. When the regulatory region was tested as a fusion protein with the Gal4 DNA-binding domain, a cryptic AD lying between amino acids 260 and 360 was inhibited by Bmh. Although about 1% of random *E. coli* sequences can display an activation function when fused to a heterologous DNA binding domain (30), the cryptic AD is only active when Bmh function is inhibited or when *ADR1*^{c} mutations are present. The specificity of its activation suggests that it may perform the same function in the context of WT, full-length Adr1, but this remains to be tested. It seems likely that the inhibitory activity of Bmh is not AD specific, because the Adr1^c region was able to inhibit activation by a heterologous activation domain (15).

The putative binding site for Bmh within the regulatory domain of Adr1 differs significantly from binding modes I and II of mammalian 14-3-3 proteins in that it lacks the well-conserved Pro at the +2 position (mode I, RXXpSXP, and mode II, RXΦXpSXP, where Φ is an aliphatic or aromatic residue, p indicates a phosphorylated residue, and X is any residue) (73). In phosphorylated peptides representing potential Bmh binding sites in yeast Nth1, which encodes a Bmh-regulated neutral trehalase, Pro was also notably absent at this position (49). It is possible that the preferred binding site for Bmh differs from the mammalian 14-3-3 consensus even though the proteins are highly conserved. Another difference is that the *ADR1*^{c} alleles span a broader range (amino acids 227 to 239) than is defined by the 14-3-3 consensus sequence. However, the length of the regulatory domain could be defined by two requirements, kinase and Bmh recognition. Thus, *ADR1*^{c} alleles could be defective in gene regulation for two reasons: reduced phosphorylation and/or reduced Bmh binding.

The evidence presented here and previously (25) suggests that Bmh acts both indirectly and directly to inhibit the activity of Adr1. Its indirect action is most likely through its role in the Reg1-Glc7 protein phosphatase complex that inactivates Snf1. Inactivation of Snf1 under high-glucose conditions is important to prevent promoter binding of Adr1 (76).

We propose, based on the work presented here, that Bmh has a direct role in Adr1 regulation through binding to phosphorylated Ser230 and inhibiting activation by an unknown mechanism. Snf1 might be intimately involved in the direct role of Bmh because it promotes dephosphorylation of pSer230 (52) and could make Adr1 a less favorable substrate for Bmh binding and inhibition. This possibility is consistent with the

Snf1-independent activation by GBD-Adr1 fusion proteins in a mutant lacking Bmh function.

The mechanism of Bmh inhibition of Adr1 activity is unknown. Nuclear exclusion is a common mechanism by which 14-3-3 proteins regulate the activity of partner proteins. However, there is no evidence that Adr1 is excluded from the nucleus under repressing growth conditions (59). The two-hybrid interaction of GBD-Adr1 with GAD-Bmh suggests that their interaction does not exclude GBD-Adr1 from the nucleus. Other alternatives include inhibition of DNA binding and inhibition of PIC formation or activity. Bmh may not be involved in promoter binding, because inhibition of this step can be overcome in a histone deacetylase mutant that has WT Bmh function (62, 70). In addition, when the Adr1 regulatory domain was fused to LexA, promoter binding occurred, as indicated by the results of an interference assay (15).

There is indirect evidence that Bmh might inhibit a post-DNA binding step in transcription activation. The Adr1-recruited, inactive PIC in a histone deacetylase mutant can be activated by substituting an Adr1^c allele for WT Adr1. As we have shown here, GBD-Adr1^c activators are Bmh insensitive. Thus, we suggest that Bmh may inhibit the activity of the poised, inactive PIC that is formed in a histone deacetylase mutant.

In conclusion, we present a model for inhibition of Adr1 activity by Bmh based on the binding of Bmh to the Adr1^c region and the inhibition of transcription activation. This mechanism of 14-3-3 protein-mediated inhibition of the activity of a transcription factor has not been described previously. The identification of the binding site for Bmh in a well-characterized region of Adr1 will allow us to study the functional and structural consequences of Bmh binding to the WT and mutant activators.

ACKNOWLEDGMENTS

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